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Fragmentation and Dispersal of the Pericentriolar Golgi Complex Is Required for Entry into Mitosis in Mammalian Cells

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Summary

The pericentriolar Golgi stacks are fragmented and found dispersed in mitotic mammalian cells. Addition of an antibody to the Golgi-associated protein GRASP65 inhibited Golgi fragmentation by mitotic cytosol in permeabilized cells. Microinjecting this antibody or the C-terminal fragment of GRASP65, which contains the antibody binding site, into normal rat kidney cells prevented entry into mitosis. Under these conditions the cells had completed S phase but were not in the prophase stage of mitosis. Fragmentation of the Golgi apparatus by nocodazole or Brefeldin A treatment prior to or post microinjection of the anti-GRASP65 antibody alleviated the block in mitotic entry. Based on our findings, we suggest that the pericentriolar Golgi organization is a sensor for controlling entry into mitosis in mammalian cells.

Introduction

The Golgi apparatus of mammalian cells is organized into stacks of cisternae. Each stack contains an average of 4–8 cisternae, and about 40–80 such stacks are attached to each other and anchored in the pericentriolar region. When mammalian cells enter mitosis, the pericentriolar stacks of Golgi cisternae undergo extensive fragmentation, and the fragments are dispersed throughout the cytosol. This process is mediated by mitogen-activated protein kinase kinase 1 (MEK1) and Polo-like kinase (Acharya et al., 1998; Sütterlin et al., 2001). A monophosphorylated form of ERK (pY-ERK) has been suggested as MEK1 effector on Golgi membranes (Cha and Shapiro, 2001). Furthermore, the putative Golgi-stacking protein GRASP55 was recently shown to be phosphorylated by the MEK1 substrate ERK2 both in vivo and in vitro (Jesch et al., 2001). Plk, on the other hand, phosphorylates GRASP65, which is a close relative of GRASP55 and is required for mitotic Golgi fragmentation in semiintact cells (Lin et al., 2000; Sütterlin et al., 2001). Thus, the pathways MEK1-ERK2-GRASP55 and Plk-GRASP65 may convert the pericentriolar Golgi stacks into small fragments during mitosis. It has also been shown that these small Golgi fragments undergo fragmentation into small vesicles or fuse with the ER (Kano et al., 2000; Shima et al., 1997; Zaal et al., 1999). Based on published results it is generally accepted that Golgi fragmentation occurs in at least two sequential steps: (1) the pericentriolar Golgi stacks are converted into small fragments (tubular reticular membranes); and (2) these fragments either undergo further vesiculation or fuse with the ER (Nelson, 2000; Rossanese and Glick, 2001).

Independent of the exact fate of Golgi membranes during mitosis, there are two issues regarding the Golgi apparatus that have gone completely unnoticed. (1) Why are Golgi membranes anchored to the pericentriolar region in mammalian cells; and (2) why do these membranes undergo fragmentation and dispersal away from the pericentriolar region during mitosis? We have addressed these questions by asking what will happen to cells in which fragmentation and dispersal of Golgi membranes are prevented at the onset of mitosis. It is conceivable that if the reorganization of Golgi membranes is prevented, cells enter mitosis and only one daughter cell inherits the Golgi membranes. The other cell either dies or generates Golgi membranes de novo. It is, however, also possible that cells do not enter mitosis if their Golgi membranes cannot be fragmented.

To address these issues, we searched for a reagent that blocks mitotic Golgi fragmentation but which by itself is not essential for regulating entry into mitosis. Protein kinases such as MEK1 or Plk are therefore not suitable candidates. Our previous findings suggested that GRASP65 could be involved in the process of mitotic Golgi fragmentation either directly or through interaction with a hitherto unidentified binding partner. We therefore decided to generate GRASP65-specific reagents (an affinity-purified antibody and a GRASP65 fragment that contains the antibody binding site) to address the connection between Golgi fragmentation and mitosis. We have found that the addition of these GRASP65-specific reagents inhibited mitosis-specific Golgi fragmentation both in permeabilized and intact cells. Most interestingly, inhibition of Golgi fragmentation using GRASP65-specific reagents prevented entry of cells into mitosis. Under these conditions, the cells have completed S phase but are not in the prophase stage of the mitotic cycle. The significance of our findings with regards to Golgi fragmentation and the regulation of entry into mitosis are discussed.

Results

Identification of GRASP65-Related Reagents that Block Golgi Fragmentation

We generated a polyclonal antiserum against the full-length epitope-tagged and bacterially expressed GRASP65 protein (Sütterlin et al., 2001). The crude anti-GRASP65 serum was affinity-purified on a GRASP65 antigen column. To test the specificity of the resulting affinity-purified antibody, we performed Western blot analysis on either purified recombinant GRASP65 protein or total

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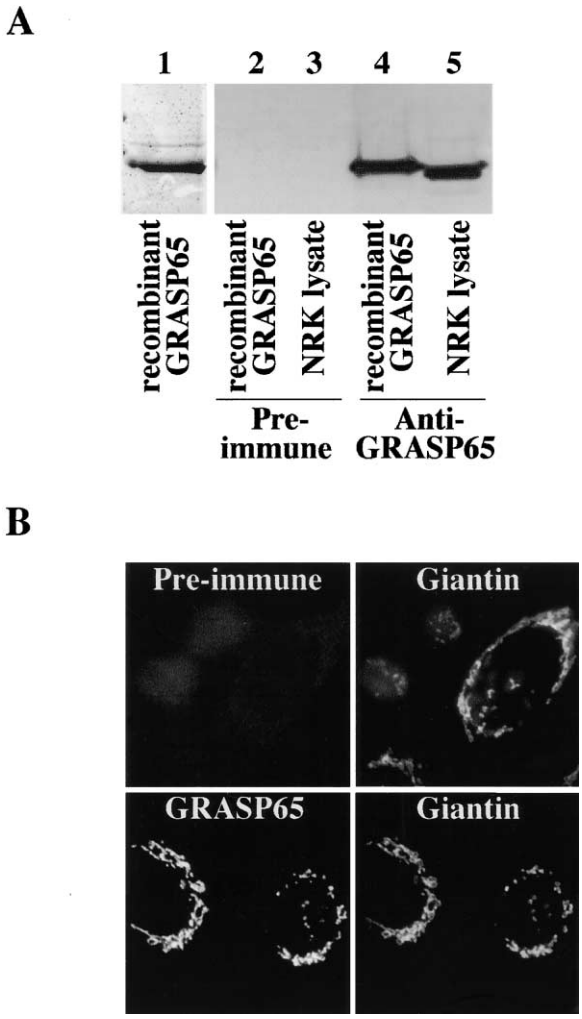

Figure 1. The GRASP65 Antibody Recognizes a Single Polypeptide in NRK Cell Extracts

(A) A construct encoding the epitope-tagged full-length GRASP65 (N-terminal 6 Histidine tag and C-terminal Flag tag) was expressed in bacteria, and the recombinant protein was purified by affinity chromatography on a Nickel-agarose column. The pure protein shown in lane 1 was used for immunization of rabbits. Preimmune serum and affinity-purified antiserum were tested for specificity by Western blotting on either purified recombinant protein (lanes 2 and 4) or total NRK lysate (lanes 3 and 5).

(B) Preimmune or affinity-purified anti-GRASP65 antibodies was tested by immunofluorescence microscopy of NRK cells. The preimmune serum does not show any specific staining. The anti-GRASP65 antibody recognizes the Golgi apparatus as determined by costaining with a bona fide Golgi protein, Giantin.

NRK cell lysates (Figure 1A). We found that the antiserum, in contrast to the preimmune IgG, recognizes picogram amounts of recombinant protein and a single polypeptide of 65 kDa in total NRK cell lysates. As expected, epitope-tagged, recombinant GRASP65 migrates slightly higher on SDS-polyacrylamide gels than the endogenous GRASP65 from NRK cells (Figure 1A, lanes 4 and 5). Immunofluorescence microscopy of NRK cells with the affinity-purified antibody and not the preimmune IgG revealed staining of the Golgi apparatus (Figure 1B).

We have reconstituted Golgi fragmentation in semiintact NRK cells (Acharya et al., 1998). NRK cells are permeabilized with digitonin, washed with 1M KCl-containing buffer, and incubated with mitotic cytosol prepared from NRK cells. After a 60 min incubation at 32°C in the presence of an ATP-regenerating system, Golgi membranes are found in the form of small punctate structures dispersed throughout the cytoplasm (Acharya et al., 1998). Electron microscopy analysis of these cells revealed that the small structures are composed of tubulo-reticular Golgi membranes (Colanzi et al., 2000).

We tested the effect of the affinity-purified anti-GRASP65 antibody in this assay. Salt-washed, permeabilized NRK cells were incubated in the presence of an ATP-regenerating system and interphase cytosol or mitotic cytosol in the absence or presence of 200 μg/ml of either affinity-purified anti-GRASP65 antibody or preimmune IgG (Figure 2A). After incubation for 60 min at 32°C, the cells were processed for fluorescence microscopy using the monoclonal anti-Giantin antibody to analyze the Golgi organization and rhodamine-conjugated anti-rabbit secondary antibody to follow the fate of the exogenously added antibodies (Figure 2A). The exogenously added anti-GRASP65 antibody recognized its target on the Golgi membranes (Figure 2A). The experiment was quantified and revealed that in the presence of the GRASP65 antibody, approximately 30% of the cells showed a fragmented Golgi apparatus compared with 65% in incubations with preimmune IgG (Figure 2B).

Based on these experiments, we conclude that the anti-GRASP65 antibody is a suitable reagent for inhibiting the fragmentation of the pericentriolar Golgi apparatus by mitotic extract (Sütterlin et al., 2001).

Addition of the C-Terminal Region of GRASP65 Blocks Mitotic Golgi Fragmentation

The N-terminal portion of GRASP65 contains both the Plk binding site (amino acids 7–120) as well as the GM130 binding site (amino acids 189–201) (Figure 3A; Barr et al., 1997; Lin et al., 2000). We first mapped the domain within GRASP65 that is recognized by the affinity-purified anti-GRASP65 antibody. Various deletion constructs of GRASP65 were generated, and the recombinant epitope-tagged proteins were expressed in *E. coli*, purified, and analyzed by SDS-PAGE followed by Western blotting (Figure 3B). We observed that, in contrast to the anti-Flag antibody, which recognized all fragments, the binding sites of the affinity-purified anti-GRASP65 antibody is located predominantly in the C-terminal region of GRASP65 (Figure 3B). GRASP65Δ200 does not contain the Plk and GM130 binding sites. We tested the effect of GRASP65Δ200 in the Golgi fragmentation process. The permeabilized cell system mentioned above was incubated with 100 μg/ml of the C-terminal region of GRASP65 (GRASP65Δ200) for 60 min at 32°C, and the organization of the Golgi apparatus was monitored by fluorescence microscopy using the anti-Giantin monoclonal antibody (data not shown). Quantitation of the assay revealed that, in the presence of the C-terminal GRASP65 fragment, Golgi breakdown occurred in 30% of the cells, in contrast to 70% in control cells lacking the GRASP65 fragment (Figure 3C).

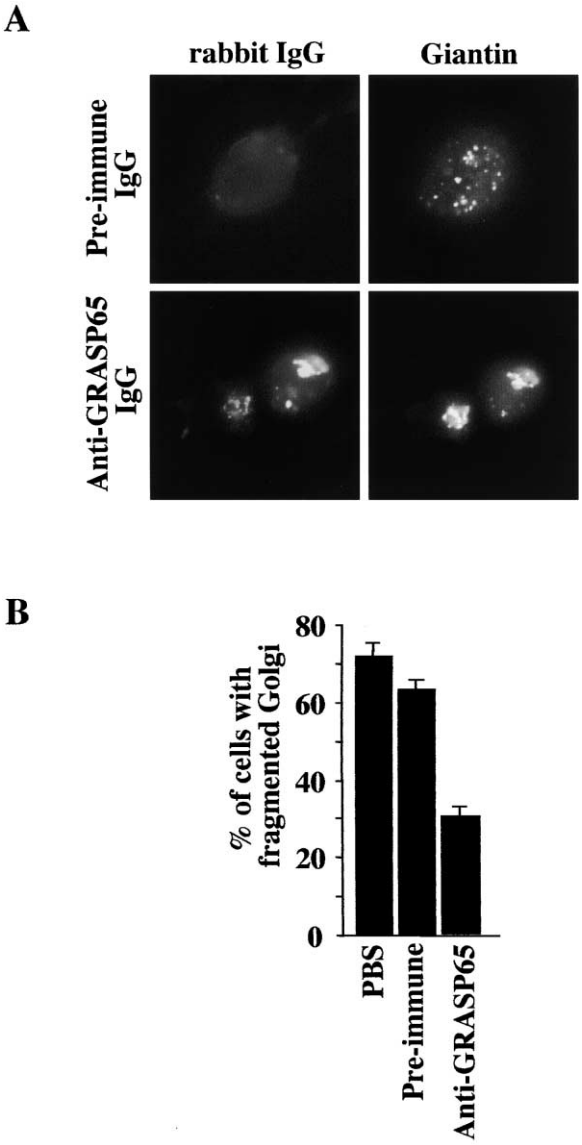

Figure 2. Anti-GRASP65 Antibody Inhibits Mitosis-Specific Golgi Fragmentation in Permeabilized Cells

(A) NRK cells were grown on coverslips and treated with 2 mM thymidine for 8–12 hr. The cells were subsequently permeabilized, washed with 1 M KCl-containing buffer, and incubated with 7 mg/ml mitotic extract in the absence or presence of 200 μg/ml affinity-purified preimmune IgG or anti-GRASP65 antibody. After a 60 min incubation at 32°C, cells were fixed and processed for immunofluorescence using an anti-Giantin antibody (Giantin) to stain Golgi membranes and rhodamine-conjugated anti-rabbit IgG (rabbit IgG) to visualize the localization of the anti-GRASP65 antibody or the preimmune IgG. The exogenously added antibody stains the same membrane fragments as the anti-Giantin antibody, suggesting that it recognizes its target protein on Golgi membranes. The presence of the anti-GRASP65 antibody inhibits fragmentation of the pericentriolar Golgi apparatus.

(B) The percentage of cells with fragmented Golgi after incubation of cells with mitotic extract containing either PBS, preimmune serum, or anti-GRASP65 antibody is shown. The data represent the average of four independent experiments.

Thus, the affinity-purified anti-GRASP65 antibody and GRASP65Δ200 inhibit Golgi fragmentation during mitosis without inhibiting and/or sequestering Plk and GM130.

Golgi Fragmentation Is Necessary for Entry into Mitosis in Intact Mammalian Cells

NRK cells were arrested in S phase by aphidicolin treatment. The cells were washed to remove aphidicolin, and at various time points after aphidicolin washout, they were stained with anti-phospho Histone H3 antibody, anti-Giantin antibody, and with the DNA-specific dye Hoechst 33342. We count a random population of 400 cells and determine how many cells have mitotic DNA and a fragmented Golgi apparatus. The number of cells in mitosis increases with time and reaches a peak of 80 at 7.5 hr after aphidicolin washout. The numbers then drop to about 13 by 9.5 hr postaphidicolin washout (Figure 4A; numbers shown are an average of two independent experiments). Mitotic cells round up and it is difficult to keep these cells attached to the coverslip, especially during the procedures for fluorescence microscopy. For all the experiments described below, we incubate cells for 7.5 hr after aphidicolin washout because at this time point the number of mitotic cells was the highest.

NRK cells were arrested in S phase with aphidicolin, washed to remove aphidicolin, and after 45 min of incubation in regular cell culture medium, 400 cells per coverslip were microinjected with either affinity-purified anti-GRASP65 antibody or preimmune IgG. At 7.5 hr after release from the S phase block, the cells were fixed and processed for immunofluorescence microscopy. Rhodamine-conjugated anti-rabbit IgG was used to detect antibody-injected cells. The organization of the Golgi membranes and the DNA was monitored by staining with the anti-Giantin antibody and Hoechst 33342, respectively. The cells were also stained with anti-phospho Histone H3 antibody (Figure 4B). Histone H3 is phosphorylated in early prophase and is thus a good marker for identifying cells in prophase and the postprophase mitotic stages (Cheung et al., 2000). Out of a population of 400 cells injected with the preimmune IgG, 74 ± 4 cells were in mitosis as judged by condensed DNA, staining with anti-phospho Histone H3 antibody, and fragmented Golgi membranes. This number is very similar to the number of mitotic cells found in noninjected control cells on the same coverslips (Figure 4C). Therefore, the antibody injection procedure per se does not have any effect on the ability of cells to enter mitosis. In contrast, out of a population of 400 cells injected with the anti-GRASP65 antibody, only 34 ± 12 cells had a fragmented Golgi apparatus and mitotic DNA (Figure 4C).

As shown above, the C-terminal region of GRASP65 inhibits the mitosis-specific fragmentation of the Golgi apparatus in our semiintact cell assay (Figure 3C). We tested the effect of this peptide on Golgi fragmentation in intact mammalian cells. The experimental protocol to test the involvement of the C-terminal region of GRASP65 was precisely the same as that described for the affinity-purified anti-GRASP65 antibody. NRK cells were treated with aphidicolin to arrest cells in S phase. The cells were washed to remove aphidicolin, and approximately 400 cells/coverslip were microinjected with GRASP65Δ200. At

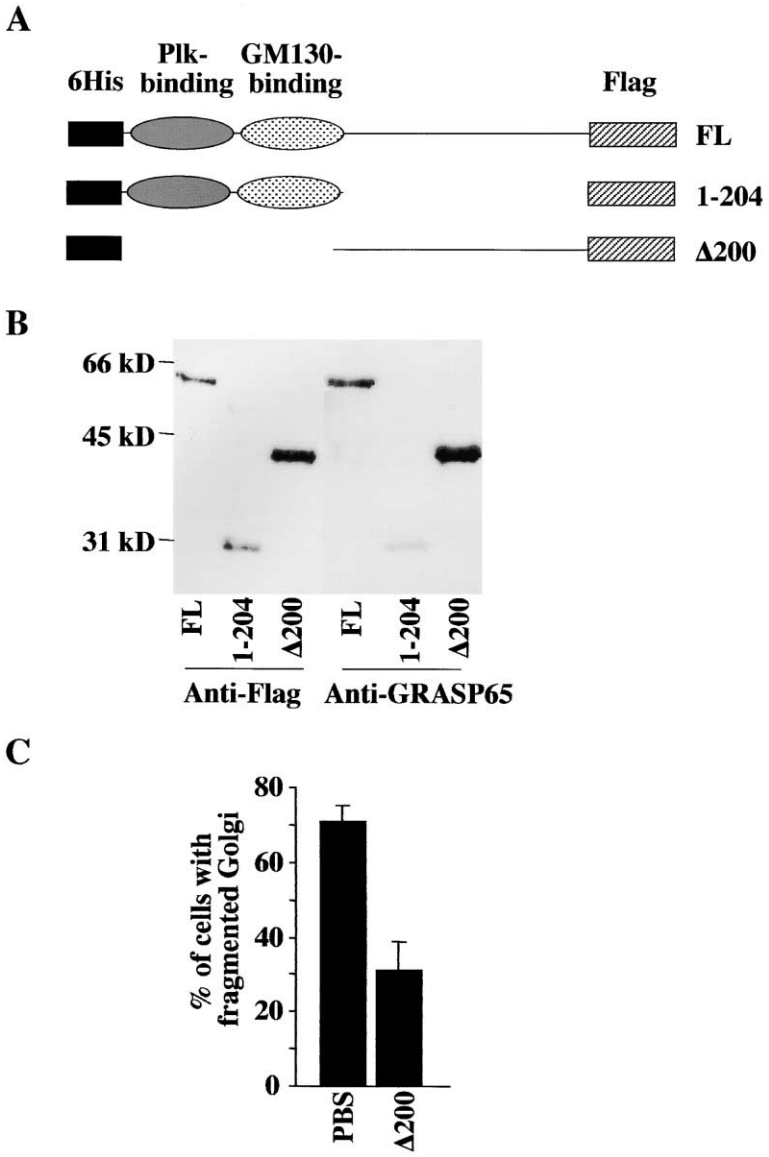

Figure 3. The C-Terminal Portion of GRASP65 Is Recognized by the Affinity-Purified Anti-GRASP65 Antibody and Inhibits Mitotic Golgi Fragmentation in Semiintact Cells

(A) A schematic drawing of the recombinant full-length GRASP65 protein (FL) and the fragments (1–204, Δ200) used in this study. All constructs contain an N-terminal 6 Histidine tag (6His) and a C-terminal Flag tag (Flag). The known binding sites for Plk (amino acids 7–120) and GM130 (amino acids 189–201) are indicated.

(B) Recombinant full-length GRASP65 (FL), the N-terminal fragment (1–204), and the C-terminal fragment (Δ200) were generated in bacteria and purified on a Nickel-agarose column. The recombinant proteins were separated by SDS-PAGE, and Western blot analysis was performed using either an antibody to the Flag epitope or the affinity-purified anti-GRASP65 antibody.

(C) The recombinant C-terminal portion of GRASP65 (Δ200) or PBS were incubated with mitotic extract and an ATP-regenerating system for 10 min. The mixtures were then added to permeabilized, salt-washed NRK cells. After a 60 min incubation at 32°C, cells were fixed and processed for immunofluorescence using anti-Giantin antibody to visualize the morphology of the Golgi. The percentage of cells with fragmented Golgi membranes in four independent experiments are shown.

7.5 hr after release from the S phase block and incubation in complete medium, cells were fixed and stained for immunofluorescence microscopy. We observed that microinjection of GRASP65Δ200 lowered the number of mitotic cells from 80 ± 10 out of a population of 400 noninjected control cells to 38 ± 5. A similar experiment was carried out with anti-GRASP65 antibody that was affinity purified against GRASP65Δ200. The cells were microinjected with this particular antibody, and it was found to inhibit Golgi fragmentation and entry into mitosis in greater than 55% of the cells (data not shown). Thus, the injection of anti-GRASP65 affinity-purified IgG or the GRASP65Δ200 peptide inhibits entry into mitosis in 50%–55% of the injected cells. Injection of the anti-GRASP65 antibody did not affect the actin and microtubule cytoskeleton (data not shown). Also, the duplication and separation of centrioles appeared normal as monitored by staining of noninjected control and anti-GRASP65 IgG-injected cells with anti-γ-tubulin antibody and pericentrin antibody (data not shown; Hinchcliffe and Sluder, 2001).

To test whether the GRASP65 antibody-mediated effect on entry into mitosis represents a delay or a block of entry into mitosis, the following experiment was carried out. Noninjected control and anti-GRASP65 IgG-injected cells were stained with DNA- and Golgi-specific reagents (described above) at various times after aphidicolin washout. For each time point, 400 cells were counted. In control cells, the number of cells with mitotic DNA and fragmented Golgi dropped from 80 at 7.5 hr to 42 at 8.5 hr and finally to 13 at 9.5 hr after aphidicolin removal. In anti-GRASP65 IgG-injected cells, the number of cells with mitotic DNA and fragmented Golgi was approximately 40 after 6.5–8.5 hr and dropped to 18 at 9.5 hr after aphidicolin washout (Figure 4E; data shown is an average of two independent experiments). In other words, while there is a sharp drop in the number of mitotic cells in noninjected control cells, the number

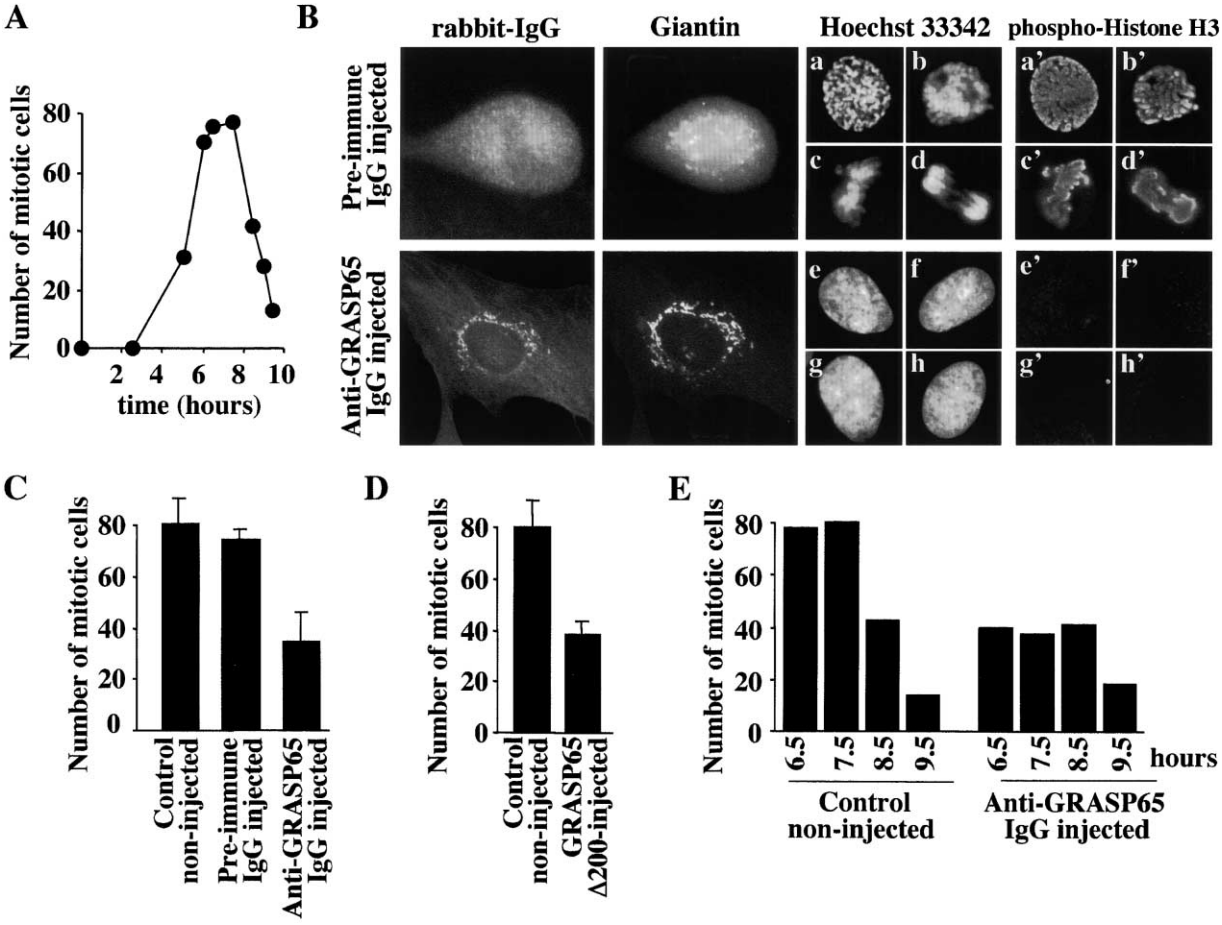

Figure 4. Microinjection of the Affinity-Purified Anti-GRASP65 Antibody or of the Recombinant C-Terminal Portion of GRASP65 Blocks Entry into Mitosis

(A) NRK cells were arrested in S phase with aphidicolin. The cells were washed to remove aphidicolin and at various times were stained with Golgi- and DNA-specific markers. The number of cells showing condensed DNA, phospho-Histone H3 antibody staining, and fragmented Golgi are shown. For each time point, 400 cells were counted. The number of cells showing these mitotic features is 80 at 7.5 hr and drops to 13 by 9.5 hr after aphidicolin washout. The data shown is an average of two independent experiments.

(B) NRK cells grown on coverslips were treated with aphidicolin for 12–16 hr. Aphidicolin was removed 45 min prior to microinjection of either preimmune IgG or affinity-purified anti-GRASP65 IgG. At 7.5 hr after aphidicolin washout, the cells were fixed and processed for immunofluorescence using rhodamine-conjugated anti-rabbit IgG to stain for injected cells, a monoclonal antibody to stain the medial Golgi marker Giantin, and the Hoechst 33342 DNA dye and the anti-phospho Histone H3 antibody to visualize the state of DNA. The cells injected with the preimmune IgG show a fragmented Golgi. These cells enter mitosis as revealed by staining with the Hoechst dye (a–d) and reactivity with the phospho-Histone H3 antibody (a′–d′). Cells injected with the anti-GRASP65 IgG contain an intact Golgi apparatus in the pericentriolar region. The staining with Hoechst (e–h) reveals that the DNA is uncondensed and there is no staining with the anti-phospho Histone H3 antibody (e′–h′). Since phosphorylation of Histone H3 is a hallmark of mitotic events beginning in prophase, these results reveal that the anti-GRASP65 IgG-injected cells have not reached the prophase stage of the mitotic cycle.

(C) Following the injection protocol described for (B), 400 noninjected control cells or cells that had been microinjected with either preimmune IgG or affinity-purified anti-GRASP65 antibody were analyzed for the organization of the DNA and the Golgi apparatus. The quantitation shows the values obtained from six independent experiments.

(D) The C-terminal portion of GRASP65 (GRASP65Δ200) was microinjected into NRK cells 45 min after their release from an aphidicolin block. FITC-Dextran was coinjected with the recombinant protein to facilitate the visualization of injected cells. Cells were left in complete medium for 7.5 hr after aphidicolin washout. The cells were fixed and processed for immunofluorescence microscopy. The Hoechst DNA dye was used to visualize the state of DNA in either noninjected control cells or cells microinjected with the GRASP65Δ200 protein. For each condition, 400 cells were counted; the values shown are derived from three independent experiments.

(E) Noninjected control cells and anti-GRASP65 IgG-injected cells were stained with the DNA dye and Golgi markers after removal of aphidicolin. The number of cells with mitotic DNA and fragmented Golgi drops from 80 at 7.5 hr to 13 by 9.5 hr in a population of 400 noninjected control cells. In cells injected with the anti-GRASP65 antibody, the number of cells with mitotic DNA and fragmented Golgi remains at about 40 at 6.5–8.5 hr and drops to 18 at 9.5 hr after aphidicolin removal. The data shown is an average of two independent experiments.

of mitotic cells in a population injected with the anti-GRASP65 IgG remains at a 50% level during this entire time period (Figure 4E). These results demonstrate that a block in Golgi fragmentation causes a block in entry into mitosis. If there had been a kinetic delay in the entry into mitosis, there should have been an increase in the number of cells with fragmented Golgi and condensed DNA in the anti-GRASP65 antibody-injected cells at the

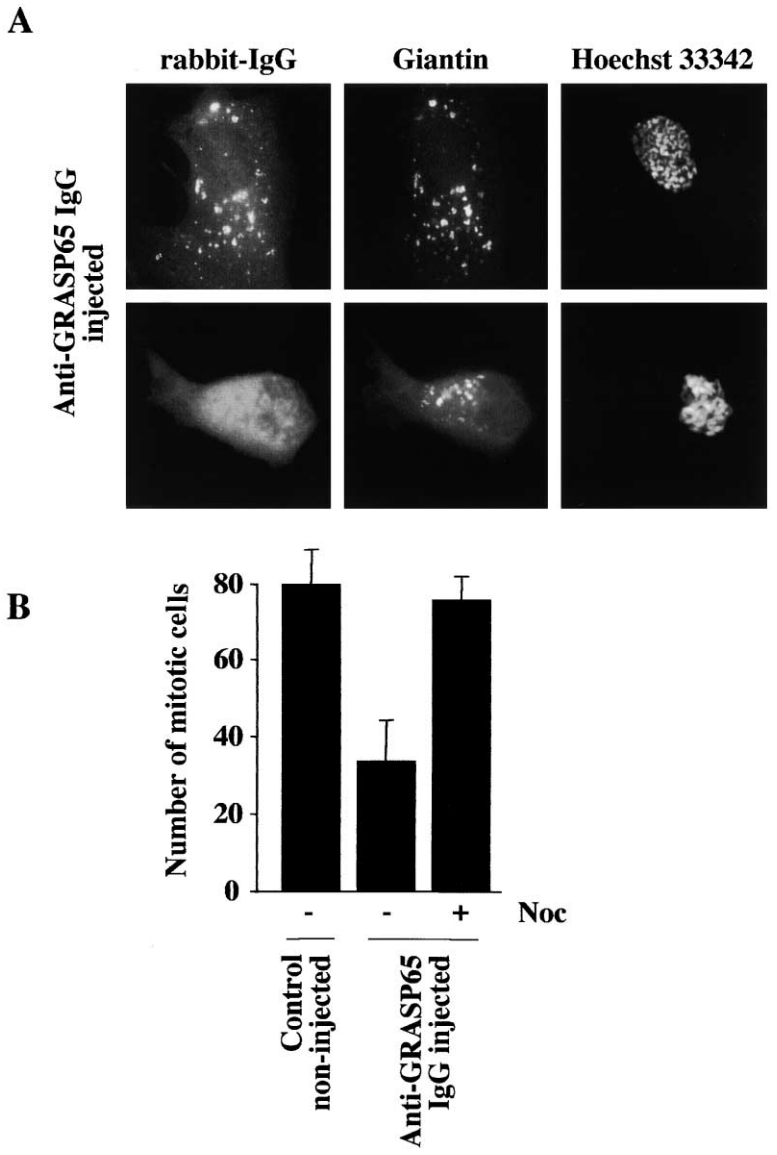

Figure 5. Prefragmentation of the Pericentriolar Golgi Apparatus Prior to Microinjection of the Antibody Abolishes the Cell Cycle Arrest

(A) NRK grown on coverslips were arrested in S phase with aphidicolin for 12–16 hr. Cells were washed to remove aphidicolin and placed in fresh medium containing 500 ng/ml nocodazole. After 90 min of treatment with nocodazole, 400 cells/coverslip were microinjected with affinity-purified anti-GRASP65 antibody. The cells that were left in complete tissue culture medium in the presence of nocodazole were fixed 7.5 hr after aphidicolin washout and processed for immunofluorescence microscopy using rhodamine-conjugated anti-rabbit IgG to detect injected cells and using anti-Giantin antibody to visualize the morphology of the Golgi apparatus. Cells were also stained with the Hoechst DNA dye. These cells enter the normal mitotic cycle as shown by the presence of cells in the early (top images) and the late stages (bottom images) of mitosis.

(B) Following the procedure for nocodazole treatment and injection of the anti-GRASP65 antibody, 400 cells were counted for each of the experimental conditions to determine the number of cells containing mitotic DNA/Golgi features. Treatment with nocodazole alleviates the anti-GRASP65 IgG-induced inhibition of entry into mitosis. The results shown are derived from four independent experiments.

8.5–9.5 hr window of this experiment. Thus, injection of GRASP65 IgG prevents entry into mitosis as evident from the findings that the cells do not contain condensed DNA nor stain with the anti-phospho Histone H3 antibody, which is a hallmark of the onset of the prophase stage of the mitotic cycle.

Fragmentation and Dispersal of the Golgi Apparatus Alleviates the Inhibition of Entry into Mitosis

Microtubule-depolymerizing agent nocodazole causes the fragmentation of the pericentriolar Golgi apparatus. As a result of this treatment, Golgi membranes are found in the form of small stacks dispersed throughout the cell (Cole et al., 1996). NRK cells were treated with aphidicolin to arrest cells in S phase. Aphidicolin was removed and the cells were treated with 500 ng/ml nocodazole for 90 min prior to injection with the affinity-purified anti-GRASP65 antibody. After 6 additional hours of incubation in complete medium containing nocodazole, the cells were fixed and processed for fluorescence microscopy as described above. In cells pretreated with nocodazole and microinjected with the affinity-purified anti-GRASP65 IgG, the Golgi apparatus appears as small fragments dispersed throughout the cell (Figure 5A). While in the absence of nocodazole, injection of anti-GRASP65 IgG caused a drop in the number of mitotic cells from 80 $\pm$ 10 to 34 $\pm$ 12 out of a population of 400 cells; the number of cells with mitotic DNA was 75 $\pm$ 8 in the presence of nocodazole. Thus, if Golgi membranes are fragmented with nocodazole prior to injection of anti-GRASP65 IgG, the anti-GRASP65 antibody does not block entry of cells into mitosis.

In another approach, we asked what happens if the cells are injected with the anti-GRASP65 antibody to inhibit Golgi fragmentation followed by fragmentation of the Golgi membranes by artificial means. Would this treatment affect entry of cells into mitosis? Treatment of cells with nocodazole depolymerizes microtubules and causes the Golgi stacks to dissociate from each

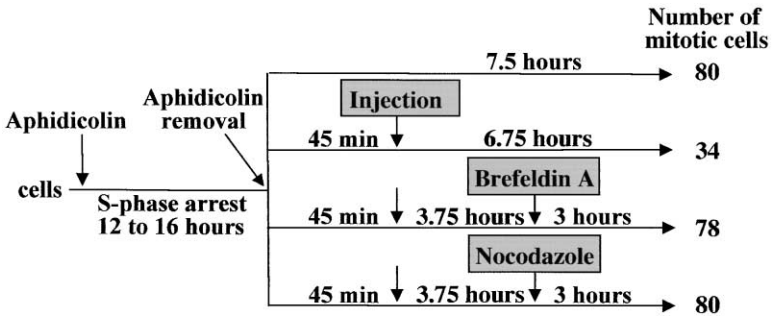

Figure 6. Fragmentation of the Pericentriolar Golgi Apparatus after Microinjection of Anti-GRASP65 IgG Abolished the Cell Cycle Arrest

NRK cells grown on coverslips were arrested in S phase with aphidicolin for 12–16 hr. Cells were washed to remove the aphidicolin and placed in fresh medium for 45 min. The cells were then injected with the anti-GRASP65 antibody. A control is included that represents noninjected control cells on the same coverslips. After 3.75 hr of incubation after aphidicolin removal, the cells was either left untreated or they were treated with Brefeldin A (BFA) or nocodazole for an additional 3 hr. The cells were then fixed and processed for immunofluorescence microscopy using rhodamine-conjugated anti-rabbit IgG to detect injected cells and using anti-Giantin antibody to visualize the morphology of the Golgi apparatus. Cells were also stained with the Hoechst DNA dye and anti-phospho Histone H3 antibody. The number of cells with mitotic DNA was determined as described in Figure 4A. Injection of the anti-GRASP65 antibody inhibits entry into mitosis, whereas the fragmentation of the pericentriolar Golgi apparatus with BFA or nocodazole alleviates the antibody-mediated inhibition of entry into mitosis. The data presented are average values from two independent experiments.

other; the stacks are then found dispersed away from the pericentriolar region (Cole et al., 1996). Treatment with Brefeldin A (BFA) causes Golgi membranes to fuse with the ER (Lippincott-Schwartz et al., 1989). NRK cells were arrested in S phase by treatment with aphidicolin. Aphidicolin was removed, and 45 min later the cells were injected with the anti-GRASP65 antibody. At 4.5 hr after release from the aphidicolin block, the cells were treated with either nocodazole or BFA for 3 hr (Figure 6). The concentrations of these drugs fragment the Golgi apparatus without any effect on the ability to enter mitosis (data not shown). The cells were then fixed and processed for fluorescence microscopy to visualize the organization of the Golgi apparatus and the DNA. As described before, out of a population of 400 cells, the number of cells with mitotic DNA was 80. Injection of the anti-GRASP65 antibody lowered this number to 34. However, in cells that had been injected with the anti-GRASP65 antibody followed by nocodazole or BFA treatment, mitotic features were found in 80 and 78 cells, respectively (Figure 6). In other words, fragmentation of the Golgi apparatus with nocodazole or BFA alleviated the anti-GRASP65 IgG-mediated inhibition of Golgi fragmentation and block of entry into mitosis.

The Block in Mitosis by Inhibition of Golgi Fragmentation Does Not Activate the DNA Damage Checkpoint Machinery

A defect in replication or artificially induced DNA damage by UV irradiation activates a set of components that arrest cells at the G2/M transition. The ATM (ataxia telangiectasia-mutated) family members are the key players in this DNA damage checkpoint, and they act by regulating Chk1 and 2, the cdc25 phosphatase and cdc2 (Abraham, 2001). Activation of the ATM pathway inhibits entry into mitosis, and this inhibition is alleviated by treating cells with caffeine (Zhou et al., 2000). To test whether cells injected with the anti-GRASP65 antibody activate the ATM pathway and are therefore arrested at the G2/M transition, the following experiment was carried out. NRK cells were treated with aphidicolin to block cells in S phase. The cells were washed to remove aphidicolin and incubated with 2 mM caffeine to prevent the activation of the ATM pathway. At 45 min after aphidicolin washout and caffeine addition, the cells were microinjected with the anti-GRASP65 antibody. After 7.5 hr of incubation, the cells were fixed and processed for fluorescence microscopy to monitor the organization of the Golgi apparatus and the DNA. The number of cells with mitotic feature out of a population of 400 cells is 36 (with or without caffeine treatment), compared with 80 mitotic cells in the population of noninjected control cells on the same coverslip (Figure 7; data shown is an average of two independent experiments). Similarly, the block in entry into mitosis by injection of the GRASP65Δ200 peptide was not alleviated by caffeine treatment (data not shown). Our findings reveal that pretreating cells with

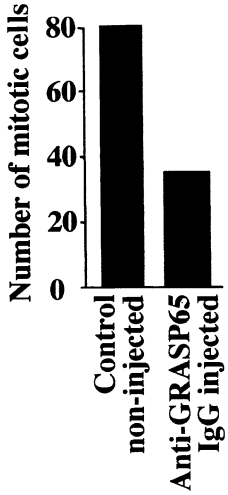

Figure 7. The Inhibition in Entry into Mitosis Is Not Due to Defective Replication or the Activation of DNA Damage Checkpoint

NRK cells were arrested in S phase with aphidicolin treatment. The cells were washed to remove aphidicolin and then treated with 2 mM caffeine. At 45 min after removal of aphidicolin and addition of caffeine, the cells were microinjected with the anti-GRASP65 antibody. After 7.5 hr of incubation in complete tissue culture medium containing caffeine, the cells were fixed and processed for immunofluorescence as described above. The number of cells in mitosis does not change significantly upon treatment with caffeine. Therefore, caffeine treatment does not override the block in entry into mitosis induced by a lack of the fragmentation of the pericentriolar Golgi apparatus. The data presented are average values from two independent experiments.

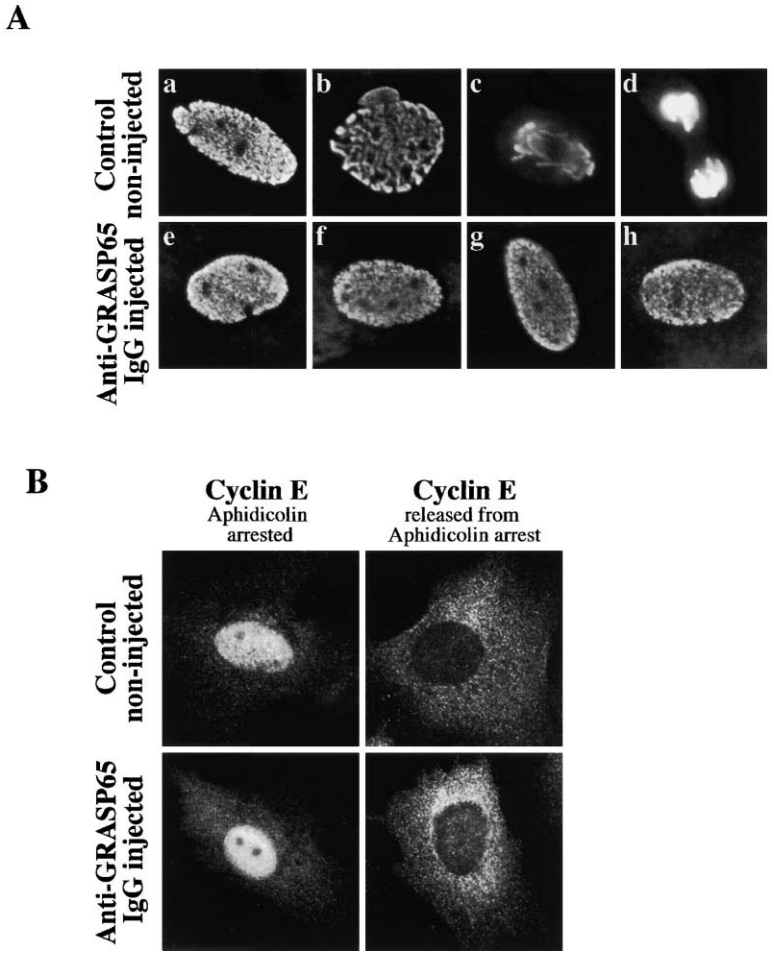

Figure 8. Inhibition in the Fragmentation of the Pericentriolar Golgi Apparatus Does Not Affect DNA Replication and the Completion of S Phase

(A) NRK cells were arrested in S phase with aphidicolin treatment. The cells were washed to remove aphidicolin and microinjected with the anti-GRASP65 antibody. Sixty minutes later, the cells were pulse-labeled for 30 min with 15 μM BrdU. The cells were fixed after 6 hr and processed for fluorescence microscopy to visualize injected cells, the organization of the Golgi, and the DNA. The noninjected control cells revealed BrdU staining of the different stages post aphidicolin removal ([a] S/post S phase, [b] prophase, [c] metaphase, [d] anaphase/telophase). The cells that are injected with the anti-GRASP65 IgG reveal BrdU incorporation into the DNA; however, these cells are not in any of the mitotic stages (e–h). Thus, BrdU uptake is not blocked by anti-GRASP65 IgG injection, but the progression into the mitotic cycle is blocked.

(B) NRK cells were arrested in S phase by aphidicolin treatment. Noninjected control cells and the anti-GRASP65 IgG-injected cells were maintained in aphidicolin for 7.5 hr. In a parallel experiment, the cells were washed to remove aphidicolin and injected with anti-GRASP65 IgG. After 7.5 hr of incubation, the coverslips were stained with anti-Cyclin E antibody. The results reveal that in aphidicolin-arrested cells, Cyclin E is predominantly within the nucleus (left images). After release from the aphidicolin block, the noninjected control cells and the anti-GRASP65 IgG-injected cells show an absence of Cyclin E from the nucleus (right images). The presence of Cyclin E in the nucleus is an indicator of the cells in S phase. By G2, Cyclin E is exported from the nucleus. Our results provide strong evidence that the anti-GRASP65 IgG-injected cells have completed S phase like their noninjected control counterparts.

caffeine and thus inactivating the ATM-dependent DNA damage checkpoint machinery does not override the inhibition in entry into mitosis caused by the block in Golgi fragmentation. This is a strong proof that the block in entry into mitosis induced by inhibition of Golgi fragmentation occurs by a novel pathway and is not through the activation of the ATM family members.

Injection of the Anti-GRASP65 Antibody Does Not Affect Replication and Completion of S Phase

In an additional experiment, cells were microinjected with the affinity-purified anti-GRASP65 antibody 45 min after aphidicolin release. After 60 min, the cells were pulse labeled with 15 μM BrdU (bromodeoxyuridine) for 30 min (Celis, 1994). Approximately 5 hr after BrdU labeling, the cells were fixed and stained with a monoclonal antibody to BrdU-stained DNA and a rhodamine-conjugated anti-rabbit secondary antibody to detect the injected cells. In noninjected control cells, BrdU is incorporated into the DNA that is uncondensed (Figure 8A, a) and into early and late mitotic DNA (Figure 8A, b–d). In cells injected with the anti-GRASP65 IgG, BrdU uptake is evident but only in the uncondensed form of DNA (Figure 8A, e–h). Thus, there is no defect in BrdU uptake in cells that have been injected with GRASP65 IgG; however, these cells are not in any of the stages of the mitotic cycle. Thus, in cells in which Golgi fragmentation was inhibited by the anti-GRASP65 antibody, there is no defect in DNA replication.

To test whether anti-GRASP65 IgG-injected cells are arrested in S phase or after completion of S phase, the following experiment was performed. Noninjected control or anti-GRASP65 IgG-injected cells were either maintained in aphidicolin-containing medium or incubated in regular medium for 7.5 hr. The cells were then stained with an anti-Cyclin E antibody. Cyclin E in S phase-arrested cells is mostly nuclear, whereas cells that have completed S phase show a nonnuclear Cyclin E staining (during this transition, Cyclin E is exported from the nucleus and ultimately degraded) (Ohtsubo et al., 1995; Yang and Kornbluth, 1999). Our results show that in noninjected control and anti-GRASP65 IgG-injected cells that are arrested in S phase, Cyclin E is

in the nucleus (Figure 8B, left images). At 7.5 hr after aphidicolin washout, Cyclin E is nonnuclear (Figure 8B, right images) in both noninjected control and anti-GRASP65 IgG-injected cells. Thus, the cells injected with the anti-GRASP65 IgG have completed S phase but are not in the prophase stage of mitosis.

Discussion

It is generally accepted that Golgi membranes in mammalian cells fragment as a consequence of entry into the mitotic cycle. Our findings reveal that the process of Golgi fragmentation is initiated prior to the appearance of these membranes in a highly fragmented form, which is observed in the early to late metaphase stage of mitosis. Preventing Golgi fragmentation at this early stage inhibits entry into mitosis. Thus, fragmentation of the Golgi apparatus is not an effect of mitosis-specific events but a key cause regulating entry of cells into mitosis. This would explain the fragmentation and the dispersal of the pericentriolar Golgi apparatus by MEK1 and Plk in the absence of cdc2 kinase in our mitotic Golgi fragmentation assay in the semiintact cells (Acharya et al., 1998; Colanzi et al., 2000; Sütterlin et al., 2001).

We have previously shown that the addition of GRASP65 prevents Golgi fragmentation in an in vitro assay reconstituting mitosis-specific fragmentation of Golgi membranes (Sütterlin et al., 2001). However, we could not rule out the possibility that this inhibition is due to sequestration of Plk, which phosphorylates GRASP65 and is required for mitosis-specific Golgi fragmentation by itself (Sütterlin et al., 2001). Here, we show that both an affinity-purified antibody to GRASP65 as well as a C-terminal fragment of GRASP65 (GRASP65Δ200), which does not contain the GM130 and Plk binding sites, prevent mitosis-specific Golgi fragmentation in vitro and in intact cells. The most likely reason for the inhibition observed with the anti-GRASP65 antibody and the recombinant protein is that they compete with a hitherto unidentified binding partner (a kinase and/or other interacting components). GRASP65 is known to interact with the Golgi protein GM130 in vitro (Barr et al., 1998). While GM130 has been suggested to play a role in mitotic Golgi fragmentation, others have reported that GM130 is not required for Golgi fragmentation and assembly during cell division in intact cells (Lowe et al., 1998; Puthenveedu and Linstedt, 2001). Regardless of the significance of the interaction between GRASP65 and GM130, we have found that the observed anti-GRASP65 antibody-dependent inhibition of Golgi fragmentation in vitro and in intact cells is not due to a block in the interaction between GRASP65 and GM130 (data not shown). GRASP65 has been reported to be required for stacking of the Golgi cisternae in vitro (Barr et al., 1997). However, there is also published data that GRASP65 continuously cycles on and off the Golgi membranes in intact cells (Ward et al., 2001). It is hard to conceive how a rapidly cycling molecule could be involved in the stacking of the Golgi cisternae, unless of course one postulates that Golgi membranes are always undergoing a process of stacking and unstacking during protein transport. Our own findings suggest a role for GRASP65 in regulating the mitosis-specific dynamics, but only when the Golgi membranes are in the pericentriolar region. Once the Golgi membranes have been fragmented and dispersed away from the pericentriolar region, for example, by treatment with BFA or nocodazole, the GRASP65-specific reagents do not affect entry into mitosis. Thus, the potency of GRASP65 inhibitory reagents vis-a-vis entry into mitosis is only effective when the Golgi apparatus is in the pericentriolar region. This is an important finding and reveals that it is the location of the Golgi membranes and not GRASP65 itself that regulates entry into mitosis.

Golgi Membranes in the Pericentriolar Region: A Sensor for Controlling Entry into Mitosis

Our finding that inhibiting Golgi fragmentation prevents entry into mitosis is both peculiar and thought provoking. We have ruled out the trivial possibility that this is due to an unspecific effect of the anti-GRASP65 antibody or the GRASP65Δ200 peptide. These reagents, when injected into cells that contain Golgi membranes distal from the pericentriolar region (as a consequence of treatment with nocodazole or BFA, for example) do not block entry into mitosis. The injection of the anti-GRASP65 antibody does not affect the organization of microtubules and the actin cytoskeleton, thus ruling out the possibility that defects in the cytoskeleton are preventing entry into mitosis.

So why should inhibiting the fragmentation of the Golgi apparatus affect entry into mitosis? There are two likely reasons for the block of entry into mitosis. (1) Fragmentation and dispersal of Golgi fragments from the pericentriolar region serves as a "specific trigger" for entry into mitosis. The rationale behind this scheme is that if Golgi membranes remain in the pericentriolar region and all else proceeds as normal, the cells will duplicate and only one of the two daughter cells will inherit the Golgi apparatus. The other cell devoid of the Golgi apparatus will not be viable, especially if the cells do not have the ability to generate Golgi membranes de novo in postmitotic cells. Therefore, the cells abort entry into the mitotic cycle. (2) The pericentriolar Golgi membranes serve as a scaffold for proteins that are required for regulating entry into mitosis. The fragmentation and dispersal of the Golgi membranes from the pericentriolar region is necessary for the delivery/transport of these key components to their respective sites of action. Implicit in this scheme is the possibility that a component necessary for promoting entry into mitosis is inactive while bound to the pericentriolar Golgi membranes. The fragmentation of Golgi membranes causes release and consequent activation of this particular component.

It is important to note that in plants, yeast, and flies, Golgi membranes are not attached to the pericentriolar region, nor do they undergo any apparent change in their organization during the cell cycle (Nebenfuhr et al., 2000; Preuss et al., 1992; Stanley et al., 1997). In contrast, the Golgi membranes in mammalian cells undergo a change from their pericentriolar nonmitotic location to a highly fragmented form in late mitotic stages. This localized pericentriolar position of the Golgi membranes may thus be an additional evolutionary advantage to control entry into mitosis. It is tempting to speculate that the preparation for entry into mitosis in mammalian

cells is governed by two distinct sets of components. One set of components monitor the status of the DNA. Defects in DNA replication (or DNA damage) activates a set of components that prevent entry into mitosis by keeping cdc2 kinase in an inactive form (Abraham, 2001). We propose the existence of a second set of components that check the status of the Golgi apparatus. Defects in the organization of the Golgi apparatus is sensed by the cells and prevents them from entering mitosis. The DNA-dependent and the "pericentriolar Golgi position-specific" controls operate independent of each other. Only when both of these events are progressing normally are the cells allowed to enter mitosis. In this manner the nuclear and cytoplasmic events coordinate to permit equal partitioning of the cellular machinery in a functional form that is necessary for growth and division of the progeny. This is a challenging proposal and identifying components that confer upon the Golgi membranes the function to act as a sensor for entry into mitosis will not be easy. But it is our hope that our in vitro assay, which is a good mimic of the situation in living cells with regards to the initiation and subsequent fragmentation of the pericentriolarly organized Golgi membranes, will help us address this issue. The use of this assay should reveal machinery that in addition to MEK1 and Plk is required for the pericentriolar Golgi fragmentation. These key reagents can then be put to test in intact cells to address the loftier proposal of "how and why" the Golgi apparatus of the mammalian cells acts as a sensor in regulating entry into mitosis.

Experimental Procedures

Reagents

The following antibodies were used in this study: anti-Giantin (Malhotra lab), anti-γ-tubulin, anti-Flag epitope (Sigma), anti-α-tubulin (Accurate Chemicals & Scientific Corp.), anti-BrdU (Calbiochem), anti-phospho-histone H3 (kindly provided by Dr. Kevin Sullivan, Scipps Research Institute, La Jolla), anti-Pericentrin (Covance), and anti-Cyclin E (kindly provided by the Newport lab, UCSD). Rhodamine-conjugated Phalloidin and Hoechst 33342 were from Molecular Probes.

Cell Culture, Preparation of Mitotic and Interphase Cytosol, and Golgi Fragmentation Assay

NRK cells were grown in complete medium (α-MEM [GIBCO-BRL] containing 10% FCS, 10 U/ml penicillin, and 100 μg/ml streptomycin) at 37°C in a 5% CO_2 incubator. Cytosol from NRK cells arrested either in mitosis or in interphase, at a concentration of 12–14 mg/ml, was prepared as described previously (Acharya et al., 1998). Permeabilization of cells grown on coverslips and the mitotic Golgi fragmentation assay was performed as described (Acharya et al., 1998). In brief, cells that have been treated with 2 mM thymidine for 8–14 hr were permeabilized on ice with 30 μg/ml digitonin in KHM buffer (25 mM HEPES-KOH [pH 7.4], 125 mM KOAc, and 2.5 mM MgOAc). Permeabilized cells were washed with 1 M KCl-containing KHM and incubated with interphase or mitotic cytosol in the presence of 200 μg/ml anti-GRASP65 antibody or preimmune IgG and an ATP-regenerating system. Incubations were carried out in a volume of 50 μl. After a 60 min incubation at 32°C, cells were fixed and processed for immunofluorescence using anti-Giantin antibody to visualize the structure of the Golgi or with rhodamine-conjugated anti-rabbit IgG to follow the localization of the antibody added to the incubation mixture. For each experiment, 200 cells per coverslip were analyzed for their Golgi morphology.

Generation of Anti-GRASP65 Antibody and Antibody Affinity Purification

Recombinant epitope-tagged GRASP65 protein was generated as described (Sütterlin et al., 2001). After the purification of 6His-tagged GRASP65 using Nickel-agarose (Qiagen), the protein fractions were loaded onto a large preparative gel for additional purification from copurifying contaminants. Pure GRASP65 was then cut out of the SDS-Polyacrylamide gel and eluted from the gel slices by electroelution and used to inject rabbits. The various bleeds were tested for reactivity with recombinant protein and total NRK lysate by Western blot analysis or by immunofluorescence. The anti-GRASP65 antibody was affinity purified as described (Harlow and Lane, 1988). In brief, an antigen column was generated by coupling approximately 4 mg of recombinant full-length GRASP65 or GRASP65Δ200 covalently to activated CNBr beads (Sigma); the column was washed with PBS and incubated with the crude antiserum. Immediately after elution of the antibody with 100 mM glycine (pH 2.5), the eluted sample was brought to neutral pH and dialyzed against PBS containing 10% glycerol in a Slide-A-Lyzer slide (Pierce). The affinity-purified antibody was then concentrated using Microcon spin columns (Millipore) to a final concentration of 12 mg/ml. Preimmune IgG was purified by binding the crude preimmune serum to a Protein A-Sepharose (Pharmacia) column. IgG was eluted with 100 mM glycine (pH 2.5), dialyzed, and concentrated as described for the anti-GRASP antibody.

Generation of Recombinant GRASP65 Fragments

GRASP65 constructs were generated by PCR using a full-length Flag-tagged cDNA construct in pBSK as template (Lin et al., 2000). PCR fragments were generated using the following oligonucleotides. Full-length, described previously in Sütterlin et al. (2001); 1–204 (upstream primer1), 5′-GGAGGCATGCGGGGCTAGGGGCAAGCAGCGAG; 1–204 (downstream primer3), 5′-GGAGAAGCTTCTACTTGTCATCGTCGTCCTTGTAGTCCTGCGTTGGGATCCGGTGCAG;Δ200 (upstream primer 3), 5′-GGAGGCATGCCCGGATCCCAACGCAGCCCTCC; Δ200 (downstream primer 1), Sütterlin et al. (2001). These PCR products were subcloned into the SphI and HindIII restriction sites of the pQE31 bacterial expression vector (Qiagen). Recombinant proteins were obtained by expression in bacteria and subsequent purification using Ni-agarose. Purified proteins were concentrated using Microcon spin columns (Millipore) and dialyzed against KHM, 5% glycerol. It is important to note that all recombinant proteins were purified in the absence of detergent.

Microinjections

Anti-GRASP65 antibody that was affinity purified to either full-length or to GRASP65Δ200 recombinant protein or preimmune IgG, all at a concentration of 12 mg/ml, were injected into approximately 400 aphidicolin-arrested NRK cells (aphidicolin was used at 2.5 μg/ml) 45 min after removal of the S phase block. Cells were then incubated in complete medium for approximately 7 hr prior to fixation. Cells were stained with anti-Giantin antibody to visualize Golgi membranes, with rhodamine-conjugated anti-rabbit IgG to visualize injected cells and with Hoechst to visualize the organization of the DNA. For the Golgi prefragmentation experiment, aphidicolin-arrested cells were washed with complete medium to remove aphidicolin and treated with 500 ng/ml nocodazole (Calbiochem) for 90 min prior to antibody injection. Golgi membranes were also fragmented after injection of the antibody by adding 200 ng/ml BFA (Sigma) or 500 ng/ml nocodazole 4.5 hr after aphidicolin washout.

Injection of the recombinant protein was performed in the same manner as described for the antibody, except that GRASP65Δ200 (at concentration of 5 mg/ml) was coinjected with Dextran-FITC (Molecular Probes) to visualize the injected cells. Cells were stained with anti-GRASP65 antibody to detect injected cells and with Hoechst 33342.

Staining of DNA with Bromodeoxyuridine

Cells were arrested with aphidicolin for 12–16 hr. At 45 min after washout of aphidicolin, the cells were microinjected with the affinity-purified anti-GRASP65 antibody. Sixty minutes later, the cells were pulse labeled for 30 min with 15 μM bromodeoxyuridine (BrdU: Calbiochem) in complete medium and fixed 7–8 hr after the initial aphidicolin removal with 4% formaldehyde, 0.5% Triton X-100 in PBS. The cells were washed with PBS, 0.05% Tween prior to denaturation of the DNA with 4 M HCl for 30 min. Cells were then washed with 0.1 M sodium borate, PBS/Tween and processed for immuno-

fluorescence using a monoclonal anti-BrdU antibody and an anti-rabbit antibody to detect injected cells.

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